WILEY-VCH

Research Article

Residue-Selective C−H Sulfenylation Enabled by Acid-Activated *S*-Acetamidomethyl Cysteine Sulfoxide with Application to One-Pot Stapling and Lipidation Sequence

Kento Ohkawachi,[a] Kaito Anzaki,[a] Daishiro Kobayashi,[a] Ryuji Kyan,[b] Takuma Yasuda,[c] Masaya Denda,[a] Norio Harada,[c] Akira Shigenaga,[b] Nobuya Inagaki,[c] and Akira Otaka*[a]

[a] Kento. Ohkawachi, Kaito. Anzaki, Daishiro. Kobayashi, Dr. Masaya. Denda, Prof. Dr. Akira. Otaka
Institute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences
Tokushima University
Tokushima 770-8505, Japan
E-mail: aotaka@tokushima-u.ac.jp

[b] Dr. Ryuji. Kyan, Prof. Dr. Akira. Shigenaga
Faculty of Pharmacy and Pharmaceutical Sciences
Fukuyama University
Hiroshima 729-0292, Japan

[c] Dr. Takuma. Yasuda, Prof. Dr. Norio. Harada, Prof. Dr. Nobuya. Inagaki
Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine
Kyoto University
Kyoto 606-8507, Japan

Supporting information for this article is given via a link at the end of the document.

Abstract: *A tyrosine (Tyr)- or tryptophan (Trp)-selective metal-free C−H sulfenylation reaction using an acid-activated S-acetamidomethyl cysteine (Cys) sulfoxide, Cys(Acm)(O), has been achieved. The dually protonated intermediate produced from the Cys(Acm)(O) under acidic conditions allows the sulfenylation of Tyr. Significantly, the reaction in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) mainly affords a Cys-Tyr-linked peptide even in the presence of Trp residues. In contrast, a Cys-Trp-linked peptide was selectively obtained from the reaction in the presence of guanidine hydrochloride (Gn·HCl) under acidic conditions. Established Tyr- and Trp-selective sulfenylation methods were used in the Cys-Tyr stapling and Trp-lipidation of glucagon-like peptides 1 in a one-pot/stepwise manner. Investigation of the mechanism showed that orbital- and charge-controlled reactions are responsible for the Trp and Tyr selectivity, respectively.*

Introduction

Post-translationally modified peptides and proteins with crosslinks between their side chains exhibit diverse signature functions by modulating the structural, electronic and spectroscopic characters of the parent molecules.[1] Among such a linkage structure, the thioether-linked 3'-(*S*-cysteinyl)tyrosine (Cys-Tyr) unit functions as the cofactor that enhances the catalytic activity of enzymes such as galactose oxidase,[2] glyoxal oxidase,[3] and cysteine dioxygenase[4] (Fig. 1a) through their interaction with redox-active metals. The biogenesis of the Cys-Tyr cofactor has been proposed to involve the tyrosyl or cysteinyl radical in the C−H sulfenylation of the tyrosine phenol side chain,[4,5] but practical and scalable synthetic access to the cofactor unit has remained challenging.[6] In research areas involving chemical modification of peptides, the arylation of Cys, and the sulfenylation of Tyr or phenylalanine (Phe) have been receiving increasing attention. These reactions generally use Pd- or Au-catalyzed reactions of aryl halide units with sulfenyl compounds.[7-10] Alternatively, aromatic nucleophilic substitution (S_NAr) of perfluorobenzenes with Cys can be utilized for the S-arylation of Cys with the intention of achieving stapling[11] between two Cys residues.[12,13] The 2'-(*S*-cysteinyl)tryptophan (Cys-Trp) unit named tryptathionine (Fig. 1b) represents another thioether-linked aromatic moiety in post-translationally modified peptides. It exhibits a unique biological function of bicyclic peptidic mushroom toxins such as Amatoxin.[14]

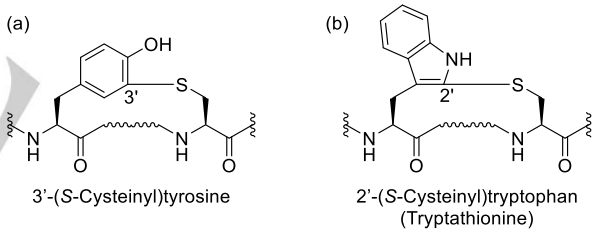


Figure 1. Structures of sp^2 (C−H) sulfenylated amino acid residues.

For the preparation of tryptathionine, oxidative coupling of oxidized Cys and Trp residues[15] and vice versa[16] has been used. We recently reported that *S*-*p*-methoxybenzyl cysteine sulfoxide (Cys(MBzl)(O))[17], an oxidized Cys derivative, participates in the reaction selectively affording tryptathionine under acidic conditions in the presence of guanidine hydrochloride (Gn·HCl) even in the presence of Tyr (Fig 2a).[18] This process includes the conversion of the acid-activated sulfoxide to *S*-chlorocysteine with release of an MBzl cation and subsequent aromatic electrophilic substitution (S_EAr) between the resulting *S*-chloride and the indole ring of Trp.

The success of the S_EAr-type sulfenylation of the Trp side chain led us to determine if an S-protected cysteine sulfoxide will allow the sp^2(C−H) sulfenylation in an S_EAr fashion of the phenol side chain of a Tyr residue in a peptide. The Cys(MBzl)(O)-enabled sulfenylation requires an ammonium chloride such as Gn·HCl to form the S-chlorocysteine and to trap the MBzl cation. The sulfenylation of Tyr is unlikely under these conditions and

consequently, we tentatively concluded that the Cys(MBzl)(O) is the wrong choice of sulfoxides for the Tyr-sulfenylation by the Cys derivatives. We therefore focused on the use of *S*-acetamidomethyl cysteine sulfoxide (Cys(Acm)(O)) (Fig. 2b).[19]

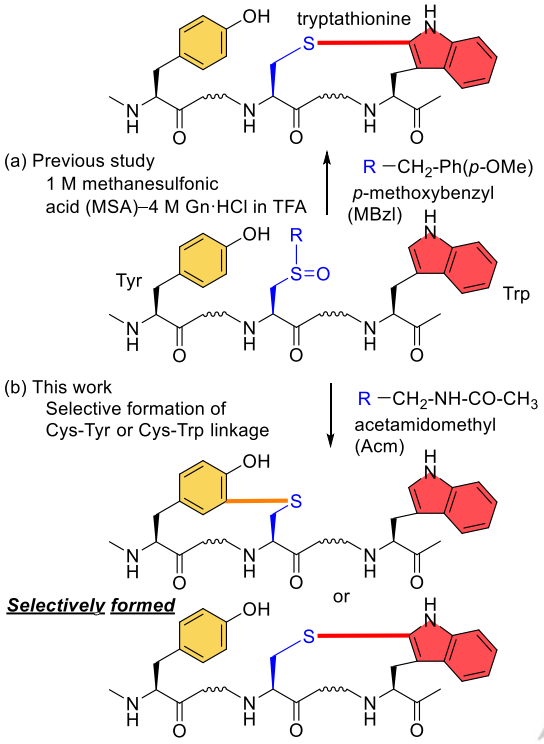


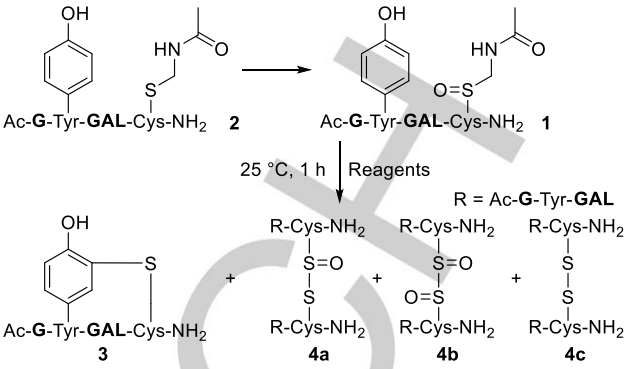
Figure 2. C−H sulfenylation of aromatic rings of Trp and Tyr. (a) Selective C−H sulfenylation of Trp (Previous study). (b) This work.

The S-Acm group in the Cys(Acm) sulfide remains intact under the acidic conditions employed in peptide synthesis,[20] but the S-MBzl group is susceptible to acid. We hypothesized that this difference in their chemical behavior under acidic conditions could allow the use of Cys(Acm)(O), instead of Cys(MBzl)(O), for the sulfenylation of Tyr. This present study has investigated the sp^2(C−H) sulfenylation of Tyr using the Cys(Acm)(O) and its evolution to the differentiation of Tyr and Trp residues and potential use in the one-pot preparation of doubly modified peptides.

Results and Discussion

Exploration of the reaction conditions suitable for the Tyr-sulfenylation began with 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) of a model peptide (Ac-**G**-Tyr-**GAL**-Cys(Acm)(O)-NH_2 (**1**)). Preliminary studies on the Cys(Acm)(O) indicated that the sulfoxide moiety is vulnerable to basic or acidic conditions used for the removal of Fmoc or side chain protections, respectively. That required the oxidative conversion of the precursor peptide (Ac-**G**-Tyr-**GAL**-Cys(Acm)-NH_2 (**2**)) to the Cys(Acm)(O)-peptide **1** with H_2O_2 in H_2O:CH_3CN:AcOH (1:1:2, v/v) (Fig. S1 in the SI). This oxidative conversion of the Cys(Acm)-peptide **2** gave the corresponding Cys(Acm)(O)-peptide **1** in a good yield (79%).

Table 1. Examination of the C−H sulfenylation of Tyr with Cys(Acm)(O) under acidic conditions.



Entry[a]	Reagents	Products (%)[b]
1	TFA	**1** (35), **3** (48), **4a** (17)
2	AcOH	**1** (>95)
3	HFIP	**1** (>95)
4	1 M MSA in TFA	**3** (>95)
5	0.1 M MSA in TFA	**3** (>95)
6	1 M MSA−4 M Gn·HCl in TFA	**3** (32), **4a** (37), **4b** (5), **4c** (25)

[a] Peptide **1** (1.0 mM) was treated with the acidic reagent at 25 °C for 1 h. The reaction was diluted fivefold with H_2O then directly analyzed by HPLC. [b] Conversion (%) (or remaining (%)) proportions were determined by HPLC analysis with UV detection at 220 nm and calculated using the equation: percent formation = 100 [(integ. **1**, **3**, **4a**, **4b**, or **4c**)/(integ. **1** + **3** + **4**)], where integ. = integration of peak area of the UV absorption. HPLC monitoring of reactions is shown in Fig. 3 and Fig. S2 in the SI. Thioether linkage at the *ortho*-position of the phenol was confirmed by NMR analyses of **3** (Figs. S3 and S4 in the SI).

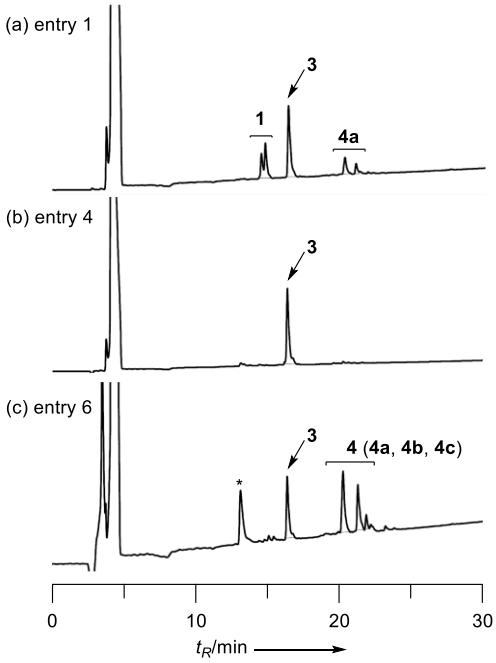


Figure 3. HPLC analysis of crude samples obtained by reactions in Table 1: (a) entry 1; (b) entry 4; (c) entry 6. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. UV detection at 220 nm. Peptides (**1** and **4a**) containing sulfoxide moieties eluted as diastereomers. *Non peptidic material.

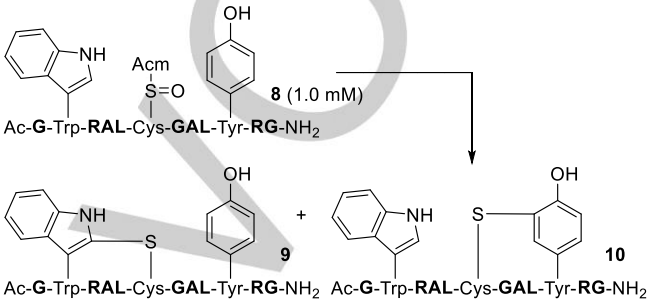
The resulting substrate **1** was then subjected to the evaluation of the C−H sulfenylation which links the Tyr and Cys residues under acidic conditions (Table 1). The reaction of **1** (1 mM) in TFA at 25 °C was incomplete after 1 h but gave a mixture of the desired *S*-aryl material **3** and the dimer peptide **4a** (entry 1, Fig. 3a). Under weaker acidic conditions such as in AcOH or hexafluoroisopropanol (HFIP), the reaction barely proceeded (entries 2 and 3, Figs. S2b and S2c in the SI). The reaction profile was dramatically changed as the acidity of the reaction increased. The reaction of **1** in 1 M methanesulfonic acid (MSA)[21] in TFA at 25 °C was complete within 10 min, giving the desired product **3** with no accompanying dimeric side products (entry 4, Fig. 3b). The reaction using 0.1 M MSA achieved a quantitative conversion (entry 5, Fig. S2d in the SI). Furthermore, the reactions of other model peptides possessing zero, one, two, four or five-residues between Cys(Acm)(O) and Tyr in 1 M MSA in TFA at 25 °C, was complete after 1 h, affording the corresponding *S*-aryl peptides in from high to quantitative yields (Fig. S5 in the SI). NMR analysis of **3** clearly indicated that the C−H sulfenylation occurs at the *ortho*-position of the phenol ring (Figs. S3 and S4 in the SI).

To learn the reason for the remarkable difference in the reactions employing TFA or 1 M MSA in TFA, we estimated the Gibbs free energy change in protonation of the model sulfoxide and amide (DMSO and *N*-methylacetamide) with MSA or TFA using density functional theory (DFT) at the B3LYP-D3/6-311+G(d,p) level in TFA using a polarizable continuum SMD (solvent model based on density) model [22]: (ΔG (DMSO with MSA) (kcal/mol) = 8.1, ΔG (*N*-methylacetamide with MSA) = 9.5, ΔG (DMSO with TFA) = 11.7, ΔG (*N*-methylacetamide with TFA) = 13.1). This result shows that dual protonation on both the sulfoxide and Acm amide moieties occurs with MSA under TFA conditions, but in TFA only monoprotonation of the sulfoxide occurs. Consequently, we hypothesized that the acidity of the reaction affects the product distribution as depicted in Figure 4.

With TFA, protonation occurs only on the sulfoxide, affording a monocationic species **5** and the subsequent liberation of the Acm cation produces cysteine sulfenic acid (Cys(OH))[23] that can behave as an electrophile and a nucleophile. An S_EAr reaction between Cys(OH) and Tyr gives the *S*-aryl unit in **3** whereas the self-condensation of the Cys(OH) unit affords the thiosulfinate type dimer **4a** which can be transformed to **4b** or **4c** by the nucleophilic attack of Cys(OH), subsequently generating Cys derivatives (Fig. S6 in the SI). On the other hand, the more acidic MSA-induced protonation involves the amide of the Acm group and the sulfoxide, and results in the formation of a dicationic species **6**. The cationic nature of the Acm moiety would prevent the S-protecting group from being liberated as a cation, and the subsequent S_EAr of **6** with the phenol of Tyr proceeds producing H_2O and affording the *S*-aryl sulfonium cation intermediate **7**. Since the Acm cation is more stable than phenyl cation, the preferential release of the Acm cation from **7** yields **3** containing the desired *S*-aryl unit.

In our previous study on the Trp-sulfenylation using Cys(MBzl)(O), ammonium chlorides such as Gn·HCl serve as an additive indispensable in the clean conversion. Similarly, the reaction of the Trp-containing Cys(Acm)(O) peptide (Ac-**G**-Trp-**GAL**-Cys(Acm)(O)-NH_2 (**S1** in the SI)) in 1 M MSA–4 M Gn·HCl in TFA quantitatively gave Trp-Cys thioether-linked peptide **S2** in SI (Fig. S7 in the SI). In contrast, the reaction of **1** in the same reagent system gave the desired **3** as a minor product and afforded instead a mixture of dimer peptides **4** as the major products (Table 1, entry 6). These results indicate that the Gn·HCl would control the reaction preference of Cys(Acm)(O) for Trp over Tyr *via* the formation of *S*-chlorocysteine that is involved in the formation of dimers in the absence of a Trp residue (Fig. S6 in the SI). Consequently, we next attempted to learn what reaction conditions contribute to the reaction preference using a model peptide containing both Tyr and Trp (Ac-**G**-Trp-**RAL**-Cys(Acm)(O)-**GAL**-Tyr-**RG**-NH_2 (**8**)) (Table 2).

Table 2. Exploration of the reaction conditions suitable for discrimination between Trp and Tyr residues in the sulfenylation using acid-activated Cys(Acm)(O).



+ mono-Acm adduct of **9** or **10** (**9** or **10** +71 Da)
+ bis-Acm adduct of **9** or **10** (**9** or **10** +142 Da)

Entry[a]	Conditions	Products (%)[b]	
		9 + **10** (Ratio 9:10)	Acm adducts + (others)
1	TFA	53 (100:0)	6[c] + (**8**, 41)
2	4 M Gn·HCl in TFA	96 (100:0)	4
3	1 M MSA in TFA	55 (45:55)	45
4	1 M TFMSA in TFA	92 (32:68)	8
5	1 M TMSOTf in TFA	78 (5:95)	22
6	1 M TMSOTf in HFIP	89 (3:97)	11
7	1 M TMSOTf−0.1 M Gn·HOTf in TFA	>91[d] (5:95)	<9[d]
8	1 M TMSOTf−0.1 M Gn·HOTf in HFIP	87 (5:95)	6 + (unidentified product[e], 7)

[a] Peptide **8** (1.0 mM) was exposed to the acidic reagent system at 25 °C for 1 h. The reaction was diluted with H_2O tenfold and directly analyzed by HPLC. [b] Conversion (%) proportions were determined by HPLC analysis with UV detection at 220 nm and calculated using the equation: percent formation = 100 [(integ. (**9** + **10**), Acm adducts or others)/(integ. **9** + **10** + Acm adducts + others)]. [c] Acm adduct of the starting material **8**. HPLC monitoring of reactions is shown in Fig. 5 and Fig. S8 in the SI. [d] Acm adducts was contaminated in the shoulder peak indicated by arrow (Fig. 5e). [e] Unidentified product possesses the MS value identical to that of **9** or **10**.

Under TFA conditions, the resulting Cys(OH) residue participated in the reaction with Trp rather than self-condensation, and gave the Cys-Trp-linked peptide **9** (Table 2, entry 1, Fig. S8 in the SI). As expected, the reaction in the presence of Gn·HCl selectively generated **9** via the formation of *S*-chlorocysteine intermediate (entry 2, Fig. 5a). These results show that both Cys(OH) and *S*-chlorocysteine prefer Trp over Tyr in the S_EAr reaction. The increase in acidity of the reaction system upon

addition of MSA or trifluoromethanesulfonic acid (TFMSA) allows the formation of the Cys-Tyr-linked peptide **10** (entries 3 and 4, Figs. 5b and 5c), which shows that the dicationic species **5** might could react with Tyr preferably over Trp. An unprecedented Tyr-selective reaction was achieved by using trimethylsilyl trifuluoromethanesulfonate (TMSOTf)[24] in TFA or in hexafluoroisopropanol (HFIP) which yielded **10** with over 9:1 Tyr-preference (entries 5 and 6, Fig. 5d and Fig. S8 in the SI).

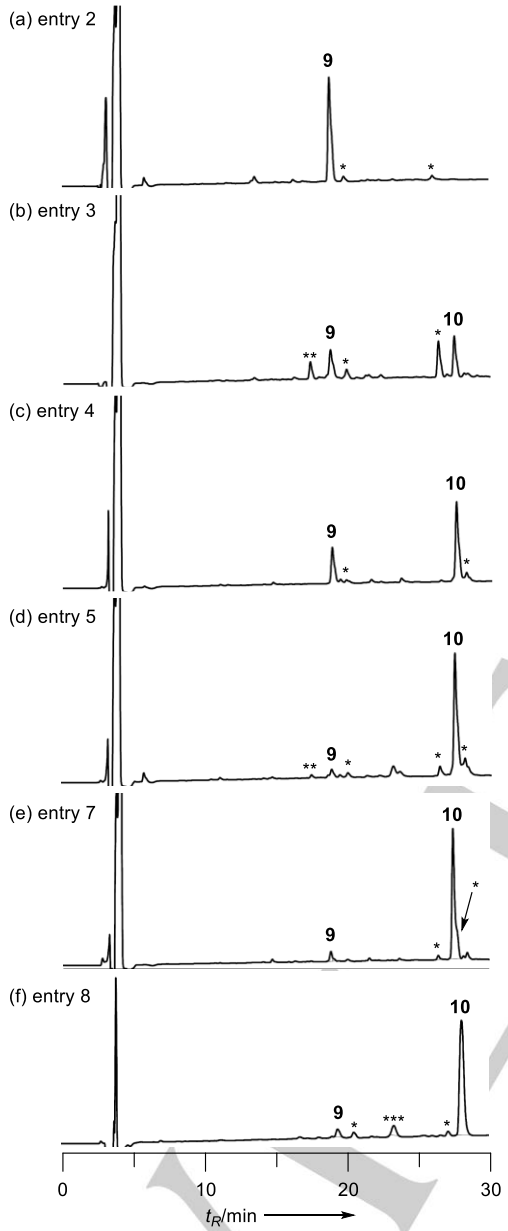


Figure 5. HPLC analysis of crude samples obtained by reactions in Table 2: (a) entry 2; (b) entry 3; (c) entry 4; (d) entry 5; (e) entry 7; (f) entry 8. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 20% to 35% over 30 min. UV detection at 220 nm. *Mono-Acm adduct of **9** or **10**. **Bis-Acm adduct of **9** or **10**. ***An unidentified product possessing the same mass identical to that of **9** or **10**.

Such a significant improvement might be attributed to the bis-trimethylsilylation of **6** which allows the dicationic species to exist long enough to react with Tyr. The reaction using TMSOTf enabled the Tyr-selective C–H sulfenylation but in this case, several (>10%) Acm adducts possessing +71 or +142 Da mass units appeared in HPLC analysis. The absence of cation scavengers in the reaction caused the alkylation by the Acm cation, but the position of the alkylation remained unclear.

To suppress this alkylation, we evaluated the cation-scavenging performance of a guanidinium salt, which can trap an iminium cation such as the Acm cation.[25] Among the guanidium salts that were examined, the guanidium trifluoromethane-sulfonate (Gn·HOTf) soluble at a concentration < 0.2 M in the TMSOTf/TFA or TMSOTf/HFIP showed satisfactory results (entries 7 and 8, Figs. 5e and 5f). Even, a decrease of TMSOTf in concentration to 0.1 M afforded the results comparable to those shown in entries 7 and 8 (Figs. S8i and 8j in the SI). Although the use of TMSOTf−Gn·HOTf in HFIP system led to formation of an unidentified product possessing the same MS value as that of **9** or **10**, the appropriate choice of the reaction solvent (TFA or HFIP) would rely on the subjected peptides as mentioned below. The reaction of **8** with 1 M TMSOTf−0.1 M Gn·HOTf in TFA for 1 h at 25 °C afforded the desired product **10** in 67% isolated yield (Fig. S9 in the SI). On the other hand, the reaction in 4 M Gn·HCl in TFA followed by HPLC purification afforded the Cys-Trp peptide **9** in 80% isolated yield (Fig. 5a and Fig. S10 in the SI). Thermolysin digestion of the obtained peptides followed by peptide mapping revealed whether the linkage was formed between Cys and Tyr or between Cys and Trp (Fig. S11 in the SI). Additionally, peptides **9** and **10** exhibited the characteristic UV-Vis absorption of the Cys-Trp[26] and Cys-Tyr[27] linkages, respectively (Fig. S12 in the SI).

The observed selectivity could be plausibly explained by the C-H sulfenylation of Tyr or Trp proceeding in either a charge- or a frontier orbital-controlled manner. The reaction affording the Cys-Trp linkage requires a neutral species such as Cys(OH) or *S*-chlorocysteine which can participate in an orbital-controlled reaction. As a Trp side chain model, 3-methylindole has a high HOMO energy level in comparison with *p*-cresol as a Tyr model. Consequently, the indole reacts with the neutral species in an orbital-controlled manner. Mulliken charge analyses of *p*-cresol and 3-methylindole showed that C2 or C6 of cresol is more negatively charged than C2 of 3-methylindole, which agrees with the chemical shifts of Tyr (3' proton: 6.61 ppm, 3' carbon: 115.49 ppm) and Trp (2' proton: 7.11 ppm, 2' carbon 124.07 ppm) (Figs. S13 and S14 in the SI). Consequently, the 3' carbon of the Tyr side chain preferentially reacts with the positively charged cation species **6** derived from Cys(Acm)(O) under the strong acidic conditions. That the silylated dication of **6** is sufficiently stable in the reaction with Tyr was confirmed by treating a Cys(Acm)(O) peptide (H-**G**-Cys(Acm)(O)-**ALFRAFG**-NH_2 (**S6** in Fig. S15 in the SI) without Tyr residue in 1 M TMSOTf in TFA at 4 or 15 °C for 20 min followed by quenching with H_2O. Under these acidic conditions, the Cys(Acm)(O) remained almost intact. On the other hand, the addition of chloride anion led to the formation of dimers through the generation of the Cys(OH), which is produced by the hydrolysis of *S*-chlorocysteine (Fig. S15 in the SI).

Next, we evaluated the applicability of the optimum conditions (1 M TMSOTf−0.1 M Gn·HOTf in HFIP or TFA) to peptides (Ac-**G**-Xaa-**RAL**-Cys(Acm)(O)-**GAL**-Tyr-**RG**-NH_2: Xaa = His (**11a**); Phe (**11b**); Lys (**11c**); Ser (**11d**); Met(O) (**11e**)) containing amino acids of concern for side reactions. With the exception of **11e**, reactions with 1 M TMSOTf−0.1 M Gn·HOTf in HFIP (1 h, 25 °C) gave the corresponding Cys-Tyr linked peptides

12 in reasonable isolated yields (His (**12a**: 71%); Phe (**12b**: 98%); Lys (**12c**: 89%); Ser (**12d**: 89%)) (Figs. S16 and S17 in the SI). The procedure for producing the Cys(Acm)(O) substrate **11e** is the same as for the oxidation of Met to Met(O), and the *in situ* reduction of the Met(O)-containing Cys-Tyr peptide by addition of TMSBr was conducted to obtain **12e** (58% isolated yield).[28] The reaction of the Ser-containing substrate **11d** required the neutral buffer treatment to reverse the N−O acyl transfer occurring in the reaction. The reactions in TFA system proceeded with a similar efficacy and are identical to those employing HFIP as solvent shown in the HPLC analyses (Fig. S18 in the SI).

With the conditions suitable for the Tyr-selective sulfenylation by the Cys(Acm)(O) in the presence of a Trp residue in hand, we next applied the residue-selective reaction to a sequence of peptide modifications including stapling[11] and lipidation[29] for improvement of the pharmacological character of the peptides. The peptide stapling features a variety of covalent linkages between side chains and has served as a promising peptide modification method for development of peptide-based therapeutic agents, and the same is true for peptide lipidation. Both modifications have advanced the therapeutic usage of glucagon-like peptide 1 (GLP-1) (7-37) as an insulinotropic peptide used to treat type II diabetes mellitus.[30,31] As a proof-of-concept experiment, we subjected the GLP-1 derivatives (GLP-1 (7-37): Gly22Cys(Acm)(O) (**13a**) and Gln23Cys(Acm)(O) (**13b**)) to a one-pot/stepwise modification consisting of the Tyr^{19}-$Cys^{22\text{ or }23}$-stapling and Trp^{31}-lipidation by Tyr and Trp-selective C−H sulfenylations, respectively (Fig. S19 in the SI). The preliminary survey of the scope of the reaction solvent, whether the TFA or HFIP is used for the reactions, indicated that HFIP is suitable for the modification of GLP-1 analogues due to the avoidance of the trifluoroacetylation side reaction. The first stapling reactions of **13** with 1 M TMSOTf−0.1 M Gn·HOTf in HFIP at 15 °C for 1.5 h proceeded in a high Tyr-selective manner to give the peptides stapled in an *i* and *i* + 3 or *i* and *i* + 4 relationship (**14a** or **14b**) without being accompanied by a detectable amount of Acm adducts. (Fig. 6a and Fig. S20 in the SI). Then, the addition of the Cys(Acm)(O)-decorated lipid **15** (1.2 equiv.) in HFIP containing 4 M diisopropylamine (DA)·HCl[30c] into the stapling reaction mixture with additional stirring at 15 °C for 1 h allowed the Trp-selective C−H sulfenylation to proceed, generating the lipidated/stapled peptides (**16a** and **16b**) in 34% and 28% isolated yields, respectively (Fig 6b and Fig. S20 in the SI). Peptide mapping of the obtained peptides using chymotrypsin indicated that the desired expected modifications occurred on the Tyr and Trp residues (Fig. S21 in the SI). The reason for using DA·HCl in the second step instead of Gn·HCl, which is indispensable for the formation of the *S*-chlorocysteine species, is that DA·HCl is more soluble in HFIP than Gn·HCl. Additionally, the mixing of Gn·HCl with the first reaction medium led to insoluble materials. The use of AcOH in the second step also resulted in the contamination of acetylation side products although the reason for the side reaction is unclear.

Subsequently, in comparison with GLP-1 (7-37) and non-stapled lipidated GLP-1 **17**,[30c] the effect of the modifications in GLP-1 peptides (**16a** and **16b**) was evaluated by an oral glucose tolerance test (OGTT) in wild-type (WT) mice. After intraperitoneal administration of phosphate-buffered saline (PBS) or the GLP-1 peptides, insulin concentrations and blood glucose were measured during the OGTT (Fig. 7). After glucose ingestion and compared to PBS, the GLP-1 peptides including non-lipidated **17** increased the insulin concentration (Figs. 7a and 7b) and reduced the glucose concentration (Figs. 7c and 7d). Blood glucose levels at 120 and 240 min were significantly lower in **16a** and **16b** than in GLP-1. Further detailed biological evaluation with structural analyses of the peptides will be in due course.

Conclusion

In conclusion, *S*-acetamidomethyl cysteine sulfoxide (Cys(Acm)(O)) enables the residue-selective C-H sulfenylation of Tyr or Trp under the appropriately selected acidic reaction conditions. A Trp-selective reaction is achieved by forming the *S*-chlorocysteine intermediate with the reaction under acidic conditions in the presence of a chloride source. In contrast, the reaction conditions allowing the formation of the intermediary dicationic species induces the C−H sulfenylation of Tyr. Significantly, the use of TMSOTf enables the high Tyr-selective sulfenylation by the Cys(Acm)(O) even in the presence of a Trp residue. Such a difference in the intermediate species could contribute to the reaction outcomes, where an orbital or charge-controlled reaction leads to the Trp or Tyr-preference, respectively. Finally, the one-pot/stepwise modification generating the doubly modified GLP-1 analogues was accomplished by the developed protocol.

Experimental Section

All experimental procedures and data are given in the Supporting Information.

Acknowledgements

This research was supported in part by AMED (20ak0101141s0101) (for A. O.). K. O. and D. K. are grateful for a JSPS fellowship (21J14744 and 21J23098, respectively).

Keywords: peptide modification • Tyr sulfenylation • Trp sulfenylation • S-protected cysteine sulfoxide • S_EAr reaction

[1] a) N. M. Okeley, W. A. van der Donk, *Chem. Biol.* **2000**, *7*, R159-R171; b) V. L. Davidson, *Biochemistry* **2018**, *57*, 3115-3125.

[2] N. Ito, S. E. V. Phillips, C. Stevens, Z. B. Ogel, M. J. McPherson, J. N. Keen, K. D. S. Yadav, P. F. Knowles, *Nature* **1991**, *350*, 87-90.

[3] M. M. Whittaker, P. J. Kersten, D. Cullen, J. W. Whittaker, *J. Biol. Chem.* **1999**, *274*, 36226-36232.

[4] C. R. Simmons, Q. Liu, Q. Huang, Q. Hao, T. P. Begley, P. A. Karplus, M. H. Stipanuk, *J. Biol. Chem.* **2006**, *281*, 18723-18733.

[5] J. Li, T. Koto, I. Davis, A. Liu, *Biochemistry* **2019**, *58*, 2218-2227.

[6] S. E. Hromada, A. M. Hilbrands, E. M. Wolf, J. L. Ross, T. R. Hegg, A. G. Roth, M. T. Hollowell, C. E. Anderson, D. E. Benson, *J. Inorg. Biochem.* **2017**, *176*, 168-174.

[7] Pd-mediated Cys arylation. a) E. V. Vinogradova, C. Zhang, A. M. Spokoyny, B. L. Pentelute, S. L. Buchwald, *Nature* **2015**, *526*, 687-691; b) A. Bruneau, M. Roche, A. Hamze, J.-D. Brion, M. Alami, S. Messaoudi, *Chem. Eur. J.* **2015**, *21*, 8375-8379; c) R. A. A. Al-Shuaeeb, S. Kolodych, O. Koniev, S. Delacroix, S. Erb, S. Nicolaÿ, J.-C. Cintrat, J.-D. Brion, S. Cianférani, M. Alami, A. Wagner, S. Messaoudi, *Chem. Eur. J.* **2016**, *22*, 11365-11370; d) A. J. Rojas, C. Zhang, E. V. Vinogradova, N. H. Buchwald, J. Reilly, B. L. Pentelute, S. L. Buchwald, *Chem. Sci.* **2017**, *8*,

4257-4263; e) H. H. Dhanjee, A. Saebi, I. Buslov, A. R. Loftis, S. L. Buchwald, B. L. Pentelute, *J. Am. Chem. Soc.* **2020**, *142*, 9124-9129; e) M. Jbara, J. Rodriguez, H. H. Dhanjee, A. Loas, S. L. Buchwald, B. L. Pentelute, *Angew. Chem. Int. Ed.* **2021**, *60*, 12109-12115; *Angew. Chem*. **2021**, *133*, 12216-12222; f) X. Chu, L. Shen, B. Li, P. Yang, C. Du, X. Wang, G. He, S. Messaoudi, G. Chen, *Org. Lett.* **2021**, *23*, 8001-8006.

[8] Au-mediated Cys arylation. a) M. S. Messina, J. M. Stauber, M. A. Waddington, A. L. Rheingold, H. D. Maynard, A. M. Spokoyny, *J. Am. Chem. Soc.* **2018**, *140*, 7065-7069; b) H. R. Montgomery, M. S. Messina, E. A. Doud, A. M. Spokoyny, H. D. Maynard, *Bioconjug. Chem.* **2022**, *33*, 1536-1542; c) G. E. Mudd, S. J. Stanway, D. R. Witty, A. Thomas, S. Baldo, A. D. Bond, P. Beswick, A. Highton, *Bioconjug. Chem.* **2022**, *33*, 1441-1445.

[9] P. Yang, X. Wang, B. Li, Y. Yang, J. Yue, Y. Suo, H. Tong, G. He, X. Lu, G. Chen, *Chem. Sci.* **2021**, *12*, 5804-5810; [1] A. J. Rojas, J. M. Wolfe, H. H. Dhanjee, I. Buslov, N. L. Truex, R. Y. Liu, W. Massefski, B. L. Pentelute, S. L. Buchwald, *Chemical Science* **2022**, *13*, 11891-11895.

[10] Recent review for metal-mediated modification of peptides, see. J. Ohata, S. C. Martin, Z. T. Ball, *Angew. Chem. Int. Ed.* **2019**, *58*, 6176-6199; *Angew. Chem.* **2019**, *131*, 6238-6264.

[11] Recent reviews for peptide stapling, see. a) Y. H. Lau, P. de Andrade, Y. Wu, D. R. Spring, *Chem. Soc. Rev.* **2015**, *44*, 91-102; b) L. Reguera, D. G. Rivera, *Chem. Rev.* **2019**, *119*, 9836-9860.

[12] A. M. Spokoyny, Y. Zou, J. J. Ling, H. Yu, Y.-S. Lin, B. L. Pentelute, *J. Am. Chem. Soc.* **2013**, *135*, 5946-5949.

[13] Review for arylation chemistry for bioconjugation including metal-catalyzed and S_NAr reactions, see. Zhang, E. V. Vinogradova, A. M. Spokoyny, S. L. Buchwald, B. L. Pentelute, *Angew. Chem. Int. Ed.* **2019**, *58*, 4810-4839; *Angew. Chem*. **2019**, *131*, 4860-4892.

[14] a) T. Wieland, *Int. J. Pept. Protein Res.* **1983**, *22*, 257-276; b) T. Wieland, H. Faulstich, *Experientia* **1991**, *47*, 1186-1193; c) J. A. Swain, S. R. Walker, M. B. Calvert, M. A. Brimble, *Nat. Prod. Rep.* **2022**, *39*, 410-443.

[15] a) M.-A. J. Siegert, C. H. Knittel, R. D. Süssmuth, *Angew. Chem. Int. Ed.* **2020**, *59*, 5500-5504; *Angew. Chem*. **2020**, *132*, 5542-5546; b) G. Yao, C. H. Knittel, S. Kosol, M. T. Wenz, B. G. Keller, H. Gruß, A. C. Braun, C. Lutz, T. Hechler, A. Pahl, R. D. Süssmuth, *J. Am. Chem. Soc.* **2021** *143*, 14322-14331.

[16] a) K. Matinkhoo, A. Pryyma, M. Todorovic, B. O. Patrick, D. M. Perrin, *J. Am. Chem. Soc.* **2018**, *140*, 6513-6517; b) C. Lutz, W. Simon, S. Werner-Simon, A. Pahl, C. Müller, *Angew. Chem. Int. Ed.* **2020**, *59*, 11390-11393; *Angew. Chem*. **2020**, *132*, 11486-11490; c) M. Todorovic, P. Rivollier, A. A. W. L. Wong, Z. Wang, A. Pryyma, T. T. Nguyen, K. C. Newell, J. Froelich, D. M. Perrin, *J. Med. Chem.* **2022** *65*, 10357-10376.

[17] S. Funakoshi, N. Fujii, K. Akaji, H. Irie, H. Yajima, *Chem. Pharm. Bull. (Tokyo)* **1979**, *27*, 2151-2156.

[18] D. Kobayashi, Y. Kohmura, T. Sugiki, E. Kuraoka, M. Denda, T. Fujiwara, A. Otaka, *Chem. Eur. J.* **2021**, *27*, 14092-14099.

[19] H. Yajima, K. Akaji, S. Funakoshi, N. Fujii, H. Irie, *Chem. Pharm. Bull. (Tokyo)* **1980**, *28*, 1942-1945.

[20] D. Veber, J. Milkowski, S. Varga, R. Denkewalter, R. Hirschmann, *J. Am. Chem. Soc.* **1972**, *94*, 5456-5461.

[21] H. Yajima, N. Fujii, *J. Am. Chem. Soc.* **1981**, *103*, 5867-5871.

[22] a) A. V. Marenich, C. J. Cramer, D. G. J. Truhlar, *J. Phys. Chem. B* **2009**, *113*, 6378-6396. b) B. R. Hashiguchi, M. M. Konnick, S. M. Bischof, S. J. Gustafson, D. Devarajan, N. Gunsalus, D. H. Ess, R. A. Periana, *Science* **2014**, *343*, 1232-1237.

[23] a) V. Gupta, K. S. Carroll, Biochimica et Biophysica Acta (BBA) - General Subjects 2014, 1840, 847-875. b) Y. Shi, K. S. Carroll, *Acc. Chem. Res*. **2020**, *53*, 20-31.

[24] a) N. Fujii, A. Otaka, O. Ikemura, K. Akaji, S. Funakosho, Y. Hayashi, Y. Kuroda, H. Yajima, *J. Chem. Soc., Chem. Commun.* **1987**, 274-275; b) N. Fujii, A. Otaka, O. Ikemura, M. Hatano, A. Okamachi, S. Funakoshi, M. Sakurai, H. Yajima, T. Shioiri, *Chem. Pharm. Bull.* **1987**, *35*, 3447-3452.

[25] B. Li, H. Tang, A. Turlik, Z. Wan, X.-S. Xue, L. Li, X. Yang, J. Li, G. He, K. N. Houk, G. Chen, *Angew. Chem. Int. Ed.* **2021**, *60*, 6646-6652; *Angew. Chem*. **2021**, *133*, 6720-6726.

[26] J. P. May, D. M. Perrin, *Peptide Sci.* **2007**, *88*, 714-724.

[27] M. M. Whittaker, Y. Y. Chuang, J. W. Whittaker, *J. Am. Chem. Soc.* **1993**, *115*, 10029-10035.

[28] N. Fujii, A. Otaka, N. Sugiyama, M. Hatano, H. Yajima, *Chem. Pharm. Bull.* **1987**, *35*, 3880-3883.

[29] Recent reviews for peptide lipidation, see. a) R. Kowalczyk, P. W. R. Harris, G. M. Williams, S.-H. Yang, M. A. Brimble, in *Peptides and Peptide-based Biomaterials and their Biomedical Applications* (Eds.: A. Sunna, A. Care, P. L. Bergquist), Springer International Publishing, Cham, **2017**, pp. 185-227; b) R. Menacho-Melgar, J. S. Decker, J. N. Hennigan, M. D. Lynch, *J. Control. Release* **2019**, *295*, 1-12.

[30] Lipidated GLP-1, see. a) L. B. Knudsen, P. F. Nielsen, P. O. Huusfeldt, N. L. Johansen, K. Madsen, F. Z. Pedersen, H. Thøgersen, M. Wilken, H. Agersø, *J. Med. Chem.* **2000**, *43*, 1664-1669; b) J. Lau, P. Bloch, L. Schäffer, I. Pettersson, J. Spetzler, J. Kofoed, K. Madsen, L. B. Knudsen, J. McGuire, D. B. Steensgaard, H. M. Strauss, D. X. Gram, S. M. Knudsen, F. S. Nielsen, P. Thygesen, S. Reedtz-Runge, T. Kruse, *J. Med. Chem.* **2015**, *58*, 7370-7380; c) D. Kobayashi, E. Kuraoka, J. Hayashi, T. Yasuda, Y. Kohmura, M. Denda, N. Harada, N. Inagaki, A. Otaka, *ACS Med. Chem. Lett.* **2022**, *13*, 1125-1130.

[31] a) P.-Y. Yang, H. Zou, C. Lee, A. Muppidi, E. Chao, Q. Fu, X. Luo, D. Wang, P. G. Schultz, W. Shen, *J. Med. Chem.* **2018**, *61*, 3218-3223; b) G. H. Bird, A. Fu, S. Escudero, M. Godes, K. Opoku-Nsiah, T. E. Wales, M. D. Cameron, J. R. Engen, N. N. Danial, L. D. Walensky, *ACS Chem. Biol.* **2020**, *15*, 1340-1348; c) Y. Yang, C. Lee, R. R. Reddy, D. J. Huang, W. Zhong, V. T. B. Nguyen-Tran, W. Shen, Q. Lin, *ACS Chem. Biol.* **2022**, *17*, 1249-1258.

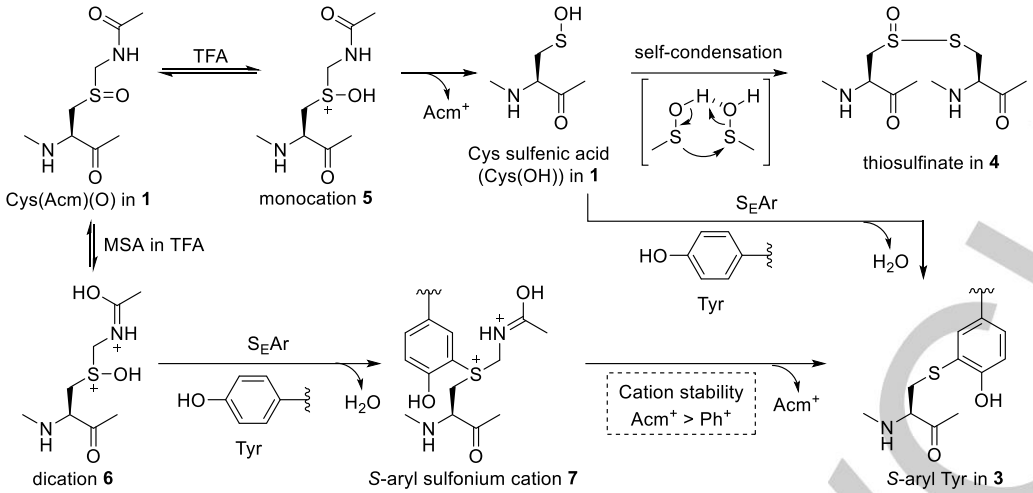


Figure 4. Proposed reaction mechanism for the sulfenylation of Tyr under acidic conditions.

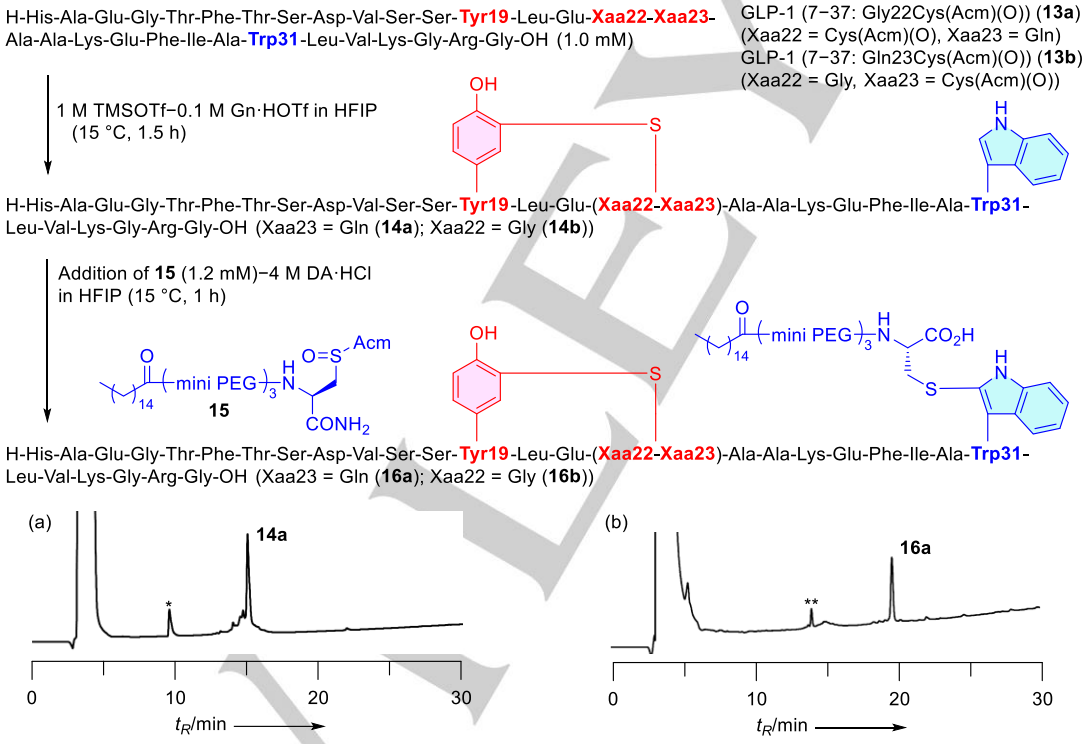


Figure 6. Scheme for the synthesis of lipidated/stapled GLP-1 **16** and HPLC analysis of the reactions affording **16a** (a) after the stapling reaction; (b) after the lipidation reaction. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5 to 95% over 30 min. UV detection at 220 nm. * Non peptidic material; **Non lipidated Cys-Trp stapled peptide.

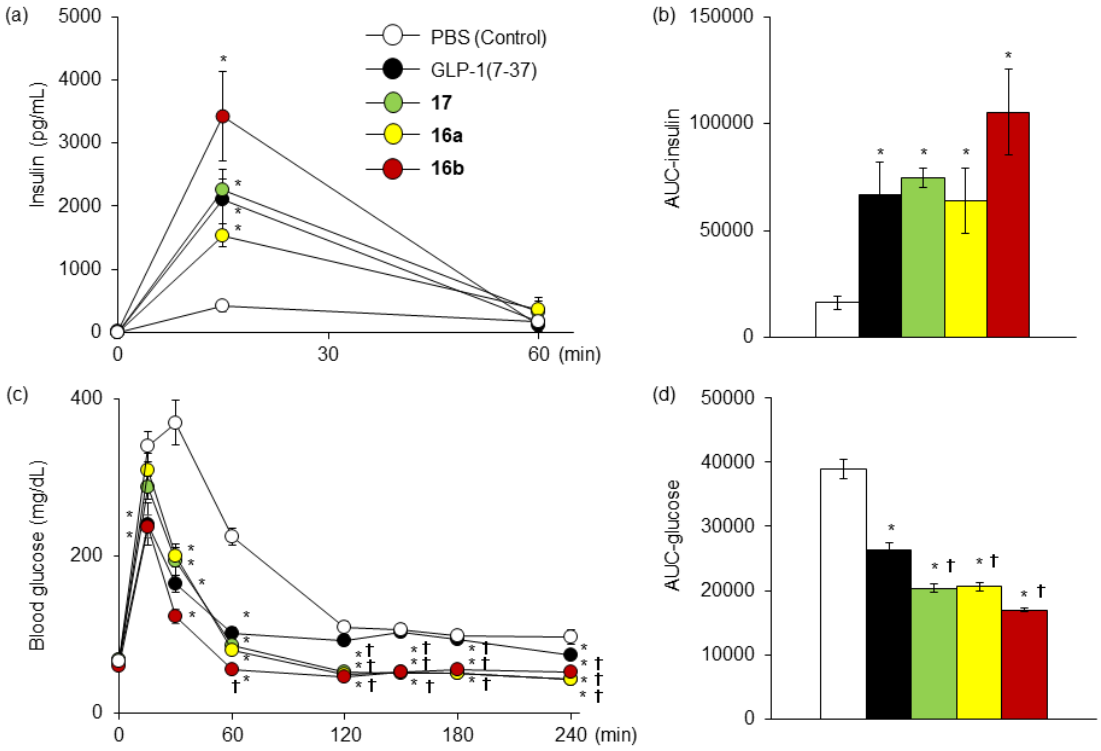
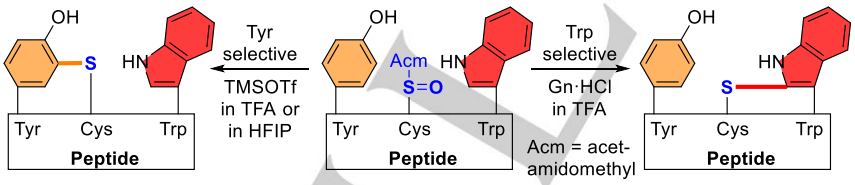


Figure 7. Blood insulin (a and b) and glucose concentration (c and d) during an OGTT in 10-week-old male WT mice (n = 7-8 mice in each group). White circles and bars indicate PBS, black circles and bars indicate GLP-1 (7-37), green circles and bars indicate **17**, yellow circles and bars indicate **16a**, and red circles and bars indicate **16b**, respectively. Statistical significance was calculated by one-way analysis of variance (ANOVA) with Tukey's test using statistical package for social science (SPSS) statistics. *P<0.05 vs. PBS, †P<0.05 vs. GLP-1 (7-37).

Entry for the Table of Contents



S-Acetamidomethyl cysteine sulfoxide (Cys(Acm)(O)) enables the residue-selective C-H sulfenylation of Tyr or Trp under the appropriately selected acidic reaction conditions. The dicationic intermediate derived from the Cys(Acm)(O) selectively reacts with Tyr, whereas the *S*-chlorocysteine allows the Trp-selective sulfenylation. The one-pot sequence of stapling and lipidation of peptides was achieved.

Institute and/or researcher Twitter usernames: @otaka_lab